Appendix A: Scope of Services

Title: Using Molecular Techniques to Preserve Genetic Integrity of Endangered Salmon in a Supplementation Program

The USFWS conducts propagation and captive broodstock programs for endangered winter-run chinook salmon at the Livingston Stone National Fish Hatchery, located at the base of Shasta Dam on the Sacramento River. The program consists of collecting adult winter-run chinook from the mainstem Sacramento River, holding and spawning the adults, rearing the juveniles in the hatchery environment, then releasing them back into the mainstem Sacramento River. Initial broodstock selection for the propagation program is critical to the maintenance of genetic integrity of the winter-run population. At the Bodega Marine Lab (BML), research will entail characterization and identification of winter-run chinook salmon through molecular and population genetic techniques. Genetic analyses are made possible by the development of microsatellite DNA markers (i.e., loci). These markers have core DNA sequences of 2-4 nucleotides that are repeated multiple times at a particular site and are transmitted via both parents, thus providing a means of assessing family origin. Microsatellites are often highly variable within populations due to a high mutation rate. They are therefore more likely to reflect recent evolutionary events and have thus been applied in a wide variety of population genetic studies, especially for closely related populations (Jarne and Lagoda 1996). Microsatellites are amplified by the polymerase chain reaction (PCR) from small, non-lethal tissue samples (caudal fin clips) and rapidly typed using denaturing polyacrylamide gel electrophoresis and fluorescent imaging (Banks et al. 1999). The genotypes of individual fish are determined for five loci: *Ots-2, -3, -9, -10* (Banks et al. 1999) and *One*∶*13* (Scribner et al. 1996). If necessary, two additional markers are used (*Ots-104* and *-107*, Nelson and Beacham 1999). The odds of a given genotype being winter run are calculated as the ratio of genotypic frequencies in winter *vs*. other runs, using frequencies for the relevant spawning populations (Banks et al. 2000) and the computer program WHICHRUN developed at BML (Banks and Eichert 2000). This program differs from traditional mixed stock analysis techniques in that it makes run probability assessments for individual fish. For winter-run fish, the log of the odds score (LOD) is 2 or greater with the core five loci (the criterion agreed by the Genetics Subcommittee 2/27/98), or 1 or greater using a total of seven loci (as discussed by the Genetics Subcommittee 2/26/99).

Objective 1. Identify individual salmon adults for use in the USFWS's winter-run chinook salmon captive propagation and broodstock programs. Salmon returning to the main stem of the Sacramento River between February through July will be trapped at Keswick Dam and RBDD, numbered and fin clipped. The tissue will then be FedExed to BML overnight. Immediately upon arrival, DNA will be extracted from each tissue sample in triplicate (Chelex technique) and amplified (PCR) at the core five loci. Using WHICHRUN, each fish will be classed as winter or non-winter run based on its LOD score. If the LOD score is less than 2, but greater than zero, the fish will be genotyped at two additional loci and the LOD score again determined. The genetic results and analyses of the trapped fish will be faxed to USFWS at Red Bluff and Livingston Stone National Fish Hatchery usually within 24 hours, but no later than three working days, after receiving the tissue

samples. We will re-evaluate the LOD score criterion for winter-run broodstock as new diagnostic loci are brought online. Computer simulations using genotypes in our extensive Central Valley chinook database (Banks et al. 2000) and the computer program WHICHLOCUS (developed at BML) will help to evaluate the likelihood that non-winter fish would be included and true winter fish excluded by any given LOD criterion. (Note: additional samples that are phenotypically WCS may be sent to BML during an extended timeframe – 2 months prior and 1 month after the February through July timeperiod (Dec - Aug).)

Objective 2. Develop new molecular markers to determine the family origin of returning hatchery-bred fish. Assigning offspring of unknown parentage to family requires a suite of highly polymorphic markers. While the core five dinucleotide loci currently used allow discrimination between winter and the other chinook runs, winter run is characterized by having markedly fewer alleles, thus making it more difficult to distinguish among winter-run families using these particular loci. However, the additional two tetranucleotide markers are more polymorphic in winter run and prove extremely useful in resolving pedigrees. Nonetheless, more polymorphic markers are required. We propose to optimize and test the Ots-200 series loci, recently developed at BML for identification of spring run, for use in winter-run pedigree analysis. We will continue to develop MHC (major histocompatibility complex) markers; we have characterized a class II gene (involved with recognition of bacterial and other extracellular antigens) and are in the process of examining a class I gene (involved with recognition of viral and other intracellular antigens). The class I gene is quite variable and should prove useful in family identification. In addition, in another project we will be using families of isogenic homozygous pink salmon to identify other genes in the MHC. We will then use the information from those screens to identify further MHC genes and their variants in winter run chinook. From this background, we will be able to determine the physical linkage relationship of these genes. We will use a computer program under development at BML (WHICHPARENT) to assign fish to family. This work is important not only in selecting non-related hatchery-bred individuals for incorporation in the captive broodstock program (of which 10% of the target capture rate is permitted), but also to confirm the effective population size model with direct estimates obtained from returning spawners. To this end, we will use this technique to make family assignments on fish collected as post-spawn winter carcasses from the main stem of the Sacramento River and Battle Creek, as well as those live trapped at RBDD and Keswick Dam.

Objective 3. Genotype and identify to run origin (e.g., winter/non-winter) salmon carcasses collected in mainstem Sacramento River or Battle Creek carcass surveys for population assessments and N_e validation. Genetic analysis of tissue will be carried out on adult carcass samples to refine run-size estimates generated in adult carcass monitoring surveys in the mainstem Sacramento River and in Battle Creek. Since DNA from carcass samples is generally degraded compared to fresh tissue, we will use the Puregene DNA Isolation Kit (Gentra systems, Inc.) to obtain higher quality DNA and perform PCR for each individual in duplicate (or triplicate, if required) as a quality control measure. We will also explore alternative sources of DNA by extracting from scales and the operculum. Individuals will be genotyped at seven microsatellite markers and analysed for run identity using WHICHRUN. Any winter run fish of hatchery origin will be assigned to family using WHICHPARENT.

Objective 4. Genotype and identify to run origin juveniles collected from rotary screw trap operations at the Red Bluff Diversion Dam or in Battle Creek for population assessments and N_e validation. Genetic analysis of tissue will be carried out on juvenile samples to refine run-size estimates generated in juvenile monitoring surveys in the mainstem Sacramento River and in Battle Creek. Tissue from juveniles will be treated with Chelex as before and genotyped at seven loci. After analyzing with WHICHRUN, the relatedness of winter-run juveniles will be determined. This will be achieved by the disequilibrium method (GENETIX v3.3, www.univ-montp.fr/~genetix/genetix.htm), although other methods, such as the computer program 'Relatedness' (http://gsoft.smu.edu/GSoft.html) and a program under development at BML (SIBLINGS), will be explored.

Objective 5. Genotype and identify to run origin returning spawners trapped in Battle Creek to determine whether a naturally spawning population of winter-run is sustained. Trapping work in Battle Creek during the same time period that fish are trapped at Keswick Dam, and determining run origin using the same genetic techniques, will enable us to verify the existence of any naturally spawning winter-run population in Battle Creek.

Objective 6. Determine genetic impacts of the supplementation program on the naturally spawning population through genetic analysis, and verify/refine an effective population size (N_e) model. To analyze the effective population size of the winter run, we will estimate the effective population size for the fish released from the USFWS winter-run chinook salmon captive propagation/broodstock program using the model developed by Hedrick et al. (1995). This will then be verified by population genetic analysis using returning spawners, both by identifying them to family and by using changes in allele frequency over multiple generations to estimate effective population size. Other approaches to evaluate N_e, such as the linkage disequilibrium approach, will be tested.

1. Justification and benefits of the project

The overriding goal of this project is to supplement the winter-run chinook salmon population and provide an insurance policy against extinction. As this project is designed to supplement an endangered population, attention to genetic considerations has remained a high priority and, since 1997, funding for genetic investigations has been sought and acquired through the Anadromous Fish Restoration Program (AFRP). The success of supplementation programs for endangered species is crucially dependent upon the maintenance of genetic variation and enhancement of effective population size. This is especially true when the size of the natural population has fallen precipitously low, such as in the case of California's winter-run chinook salmon (*Oncorhynchus tshawytscha*). Few hatcheries have integrated molecular genetics with an artificial rearing program. However, since 1998, we have used molecular markers developed at the Bodega Marine Laboratory (UC Davis) to aid in selecting winter-run broodstock for a supplementation program at Livingston Stone National Fish Hatchery. We have developed a rapid procedure for identifying winter-run individuals, trapped at the barrier dam on the main stem of the Sacramento River during broodstock harvest, to ensure that they are not inadvertently hybridized with conspecific runs. In addition, by using microsatellite markers to identify the families of returning spawners, we can document the population dynamics of hatchery-spawned salmon and assess directly their genetic impact on the naturally spawning population.

Conceptual model

The impact of hatchery supplementation on genetic diversity is mediated through effects on the effective size (N_e) of the natural population. N_e determines the rate at which deleterious mutations are fixed through the process of random genetic drift, reducing fitness and increasing chances of population extinction. N_e is a theoretical construct, the size of a mathematically ideal population that has rates of genetic drift and inbreeding equivalent to those in an actual population under study. In the mathematically ideal population, there are equal numbers of both sexes, adults mate at random, and variance in number of offspring per adult is binomial or Poisson. The number of adults N in the ideal population is, by definition, equal to the effective size, and the ratio of $N_e/N = 1.0$ in the ideal case. In actual populations, the sexes may not be in equal numbers, mating may not be at random, or the variance in offspring number may be larger than binomial or Poisson. Consequently, the N_e/N ratio for most vertebrate populations is thought to lie between 0.25 and 0.75 (Nunney 1992).

Conservation biologists have discussed a number of minimum effective population numbers. From the standpoint of protecting against inbreeding depression, which increases at a rate of $1/2N_e$ per generation, effective sizes of 50 and above would appear to be sufficient (Franklin 1980). However, to avoid long-term loss of variation or to conserve rare alleles that might be the basis of future adaptation, effective sizes above a minimum 500 or even 5000 may be needed (Franklin 1980; Lande and Barrowclough 1987). Traditionally, N_e has been difficult to estimate for natural populations, although a variety of methods has emerged in the past decade (Waples 1991; Pudovkin et al. 1996; Luikart and Cornuet 1999). For a hatchery-supplemented population, N_e depends on the effective sizes of the hatchery and wild components of the population and on the relative proportion of hatchery origin fish (Ryman and Laikre 1991):

$$N_e = \frac{N_{eh} \times N_{ew}}{x^2 N_{ew} + y^2 N_{eh}}$$

N_{eh} and N_{ew} are the effective sizes of the hatchery and wild components of the population, respectively, and x and y are their relative contributions to the total ($x + y = 1.0$). In this model, only three independent parameters are necessary to describe the impact of hatchery enhancement on natural biodiversity. Thus, a simple theory is available for evaluating the genetic impact of hatchery supplementation, reducing uncertainty of hatchery operation and providing the baseline data necessary to guide annual management decisions. The cumulative high-quality, long-term database this project provides (in tandem with other concurrent genetic stock assessments) will underpin the ecosystem-wide assessment of Central Valley chinook salmon. Without these data it is impossible to assess population integrity either biologically or legally under the ESA. Only through the use of molecular genetic analysis can hatchery intervention support the preservation and enhancement of wild stocks. This model links assessment and intervention as a preliminary step for future hatchery practice.

2. Monitoring and data evaluation

Using molecular markers to identify winter-run chinook is critical for the effective management of endangered salmon populations. The molecular techniques we have developed enable us to conduct

rapid response genetic analysis on returning spawners to determine their run origin. This procedure is vital to prevent inadvertent hybridization between genetically distinct runs, an error that occurred prior to 1998 before the inclusion of genetic factors in broodstock selection. It is imperative to continue using these techniques to protect the genetic integrity of this endangered population until the natural run has increased sufficiently and hatchery supplementation is no longer required.

Development of new molecular markers will strengthen efforts to assess the effectiveness of winter-run supplementation. Different numbers and kinds of molecular markers are needed for different tasks. For example, assignment to winter run can be accomplished with an existing set of five to seven moderately polymorphic markers. However, determining parentage requires several highly polymorphic markers, and estimating average pairwise disequilibrium has an inherently high variance that can only be reduced by using as many markers as possible. Our ability to assign individuals to hatchery family origin using genetic techniques is dependent upon the type of molecular markers used. The more polymorphic the marker, the more likely different families will have unique alleles enabling them to be more easily distinguished from one another. Increasing the inventory of markers will reduce uncertainty in genetic assessments, increasing the positive feedback mechanism in the adaptive management framework.

Molecular markers will determine whether a naturally spawning population of winter run exists in Battle Creek. Due to year-round cold water springs originating from Mount Lassen, Battle Creek has the potential to support a naturally spawning winter-run population. By trapping and genotyping returning individuals, we can ascertain whether any non-hatchery individuals (identified by the absence of an adipose fin clip) are winter run and estimate the size of this population.

Assigning returning spawners to families created in the hatchery and improving run-size estimates of the natural population enables direct estimates of the effective population size of the hatchery release. Hedrick et al. (2000b) have demonstrated that the predicted effective population sizes of the 1994 and 1995 hatchery releases are remarkably close to the direct estimates obtained from the returning spawners. Long-term estimates provide the opportunity to test the performance of a predictive model in an empirical context. These estimates are critical to the evaluation of the potential genetic impact of the artificial propagation program on the natural population (Hedrick et al. 1995, 2000a). Long-term data are required to determine whether differential survival occurs among returning families. Because salmon return to spawn between two and four years of age, at least three years are required to adequately assess the return rate of a single cohort. In addition, year-to-year variation in environmental conditions (e.g., El Niño, La Niña), which influence both the age and number of returning spawners, necessitate data collection on a longer time scale. Refining winter-run size estimates of the natural population by genotyping adult carcass returns and outmigrating juveniles will also lead to increased predictive power of the effective population size model.

3. Work to be performed and deliverables

Task 1. February -July, years 1 and 2.

Genotype all adult chinook salmon trapped at Keswick Dam and RBDD for potential use in the USFWS artificial propagation program ("Rapid response" genetic analysis). All samples to be

analysed at 5 loci, or 7 loci if 0<LOD<2. (Note: additional samples that are phenotypically WCS may be sent to BML during an extended timeframe – 2 months prior and 1 month after the February through July timeperiod (Dec - Aug).)

Task 2. October 2001–September 2003.
Develop additional polymorphic markers (e.g., Ots-200 series, MHC class 1 and II) to aid in family origin and linkage disequilibrium analyses. Determine parentage of returning spawners of hatchery-origin to verify effective population size predictions.

Task 3. October–January, years 1 and 2.
Genotype carcasses obtained in the Sacramento River and Battle Creek between April–August 2001 and 2002. Estimate the proportion of winter-run chinook, determine the parentage of hatchery-origin fish to verify N_e predictions, and assess temporal N_e variation. Test alternative DNA sources to fin tissue, such as scales and opercula.

Task 4. May–September, years 1 and 2.
Genotype a subsample of outmigrating juveniles caught at RBDD and Battle Creek screw traps in July–December 2001 and 2002. Verify the proportion of winter-run chinook, verify non-relatedness, and estimate the N_e of the natural population through linkage disequilibrium analyses.

Task 5. February–June, years 1 and 2.
Genotype all adult chinook salmon trapped in Battle Creek to determine any naturally-spawning winter run. All samples to be analyzed at 5 loci, or 7 loci if 0<LOD<2.

Task 6. October 2001–September 2003.
Computer simulations using WHICHRUN and WHICHLOCI to test winter/non-winter assignment probabilities with additional loci. Refinement of computer program WHICHPARENT to aid in assigning returning hatchery-bred individuals to family. Development of computer program SIBLINGS to assess relatedness among juvenile samples.

Task 7. Arizona State University subcontract. October 2001–September 2003.
Use genetic analyses from Tasks 2, 3 and 4 to verify the effective population size model and thus monitor potential genetic impacts of the artificial propagation program on the natural population.

Project Management. Ongoing. Provide status reports on completed work and research advances to AFRP/USFWS. Prepare publications for refereed journals and presentations at scientific meetings.

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